HEAT STABILITY OF 3-ISOPROPYLMALATE DEHYDROGENASE: DENATURATION-RENATURATION STUDIES

Ph. D. Thesis Booklet

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Introduction

Formation of the native molecular structure is crucial for proper functioning of proteins, including enzymes. In the native three-dimensional structure the linear polypeptide-chains that are held together by covalent interactions, in most cases, are folded into a globular form. This form is **stabilized by H-bonds, salt bridges and hydrophobic interactions** operating among the side-chain and backbone atoms of amino acid residues. By this way the residues, that are far in the primary structure, can be positioned close to each other in the three dimensional structure. A very early experiment with ribonuclease, performed by Anfinsen, clearly proved that the primary sequence unambiguously determines the native spatial structure. However, the route from primary to the higher levels of the structure could not be described in detail and understand properly.

Structural stability of proteins is closely related to their three dimensional structure. Proteins are widespread even under extremely different environmental conditions and the same protein from organisms living at different temperatures possesses different structural stabilities. The **structural and energetic origin of** differences in structural **stability** – in spite of numerous comparative studies – is not yet understood.

My Ph. D. work was aimed to unreveal the **denaturation-renaturation mechanisms of the 3-isopropylmalate dehydrogenases (IPMDH) and the possible relationship with their different heat stabilities**. IPMDH is a homodimer, two-domain enzyme that catalyzes the third step of leucine biosynthesis. The folding mechanism of oligomeric proteins is generally very complex, scarcely investigated, and therefore barely understood. Investigation of IPMDH was also expected to clarify the role of the subunits and domains in formation of the native structure.

Concerning the **role of subunits** I investigated the question whether subunits of IPMDH could fold independently or could assist in folding of each other; namely whether association of polypeptide-chains takes place in the early stages of folding or only the subunits with native structure are able to associate.

As for the **role of the domains** in the folding process I planned to clarify whether the domains of IPMDH are able to form their native structure individually or the native conformation can only be reached in a highly cooperative manner.

Studying IPMDH provided possibilities to unreveal any general relationship between heat stability and folding. This knowledge would promote planning new proteins with high structural stability for industrial utilization, purely on the basis of their sequence.

Aims of the work

My Ph. D. work was aimed to clarify the denaturation-renaturation mechanism of IPMDHs from the thermophilic *Thermus thermophilus* (Tt), mesophilic *Escherichia coli* (Ec) and psychrotrophic *Vibrio* sp. I5 (Vib) organisms. These enzymes were chosen as a model-system to investigate the question whether folding of proteins with different structural stabilities follow the same or different pathways. A further general question is whether domains, isolated subunits or the associated polypeptide chains are the minimal structural units that possess all information required for formation of the native structure.

For these purposes the following experimental plan was prepared:

1. To explore the renaturation-denaturation mechanism of the three IPMDHs with different heat stability by means of **equilibrium measurements**.
2. To determine the **kinetic mechanism** of both unfolding and refolding of IPMDHs with different heat stabilities. I planned to follow these processes by monitoring changes in enzyme activity, thiol-reactivity and the spectral signals of CD and fluorescence, both in the presence and absence of the substrates. To interpret the results comparative analysis of IPMDH sequences and the available crystal structures of the thermophilic, mesophilic and psychrophilic (psychrotrophic) enzyme forms are also aimed.
3. The **characterisation of the folding intermediate** based on its spectral properties, oligomerisation state and substrate binding abilities, etc..
4. To detect the presence of **subunit** as an assumed folding intermediate fluorescence anisotropy measurements and kinetic analysis at different protein concentrations are devised.
5. For separate monitoring of the refolding time courses of each **domain** the following site-directed Trp mutants were prepared and their refolding was investigated as described above using various biophysical technics:
   - W152F – refolding can be followed without contribution of the arm-region
   - W152,77F – refolding of the 2. domain can be detected separately
   - W152,195F – refolding of the 1. domain can be monitored separately.
6. I expected to provide adequate **structural explanation** of the extremely different unfolding as well as the closely similar refolding rates of IPMDHs by analysing the atomic contacts on the subunit and domain interfaces as given by the crystal structures of Tt, Ec and Vib enzymes.

Experimental methods

IPMDHs from *Thermus thermophilus* (Tt) and its Trp mutants as well as from *Escherichia coli* (Ec) and *Vibrio* sp. I5 (Vib) were expressed in *E. coli* cells.

Equilibrium denaturation experiments were carried out by monitoring **CD- and fluorescence spectral changes** and by means of **urea-gradient gelelectrophoresis**.

The **time courses** of both denaturation and renaturation were followed by registration of the changes in CD- and fluorescence spectra and in **enzyme activity**. In cases of the thiol-containing Ec and Vib IPMDHs unfolding was also detected by measuring **thiol-reactivity**. To characterize the nature of the intermediate(s) - in addition to earlier mentioned methods – I also applied the phenomena of **ANS fluorescence** and **Förster Resonance Energy Transfer** (FRET).

Kinetic analysis of refolding, followed by detecting the changes of Trp fluorescence, were carried out at different protein concentrations in order to explore whether association of the two polypeptide-chains takes place in the course of refolding. The same question was also investigated by following the changes of the **fluorescence anisotropy** during renaturation.

Similar refolding studies were carried out with various simple and double Trp mutants of Tt IPMDH, designed with the aim of separate investigation of refolding process of each domain. Conformation of the mutant proteins was tested by **differential scanning calorimetric** (DSC), **CD spectroscopic** measurements and **native gelelectrophoresis**.

For visualisation and analysis of the available high resolution crystal structures of IPMDH the **molecular graphical** software Insight II (Biosym/MSI) was used. During such analysis the conserved residues were identified by aligning all available 272 IPMDH sequences using the ExPASy Molecular Biology server. The important atomic contacts of the conserved residues responsible for stabilization of the relative positions of various secondary structural elements are envisaged by molecular graphics. The role of non-conserved side-chains and their contacts in stabilization of the heat resistant forms were also investigated. Furthermore, tertiary contacts at the domain-domain and subunit-subunit interfaces were analysed using the same method.

New scientific results

The objects of my Ph.D. studies were 3-isoproylmalate dehydrogenases (IPMDHs) with different heat stabilities. The molecule of all IPMDHs consists of two identical subunits and each of the subunit contains two structural domains. The IPMDHs were considered as models in investigating two different, but close related questions:

1. What are the role of subunits and domains in the self-organization process of the three-dimensional structure of proteins?
2. What is the relationship between the folding mechanism and the heat stability of proteins?

The main results are the followings:

1.

Equilibrium denaturation experiments, using CD-, fluorescence spectroscopy and ureagradient gelelectrophoresis, revealed that the differences in stabilities of Tt (thermophilic), Ec (mesophilic) and Vib (psychrotrophic) IPMDHs can not be simply attributed to the values of the denaturation free energy changes (ΔG^0). While **the transitions between folded and unfolded states** of Ec and Vib IPMDHs are unambiguously **complex** processes and can be described by a three state model, these experiments with Tt enzyme do not confirm the complexity of the folding. However, the complex nature of the mechanism, including Tt IPMDH, has revealed by further kinetic refolding experiments presented below.

2.

To determine the **kinetic mechanism** of refolding for IPMDHs with different heat stabilities – besides the CD- and fluorescence spectroscopy methods – I also applied enzyme activity tests and in the cases of Ec and Vib IPMDHs thiol-reactivity measurements. It was found, that the **denaturation of all studied IPMDHs** is an apparently **single first order process**, however their **rate constants significantly deviate** from each other. Denaturation of the thermophilic enzyme is the slowest, while that of the psychrotrophic Vib enzyme is the fastest process. The reverse process of **renaturation** was established to be a **complex process** for all of the three IPMDHs: it consists of a burst and a much slower first order phase. The **rates of the whole renaturation process are not significantly different** from each other for the three IPMDHs.

The time courses both denaturation and renaturation were also investigated in the presence of the substrates. The rate of denaturation decreased significantly in the Mn*IPM binary complex as well as in the functioning (NAD*Mn*IPM) and non-functioning (NADH*Mn*IPM) ternary complexes. On the other hand, the bound NAD or NADH had no any effect on the unfolding rates. Thus, the bound **Mn*IPM** is clearly responsible for reduction of the unfolding rate, i.e. it **protects the polypeptide-chains against denaturation**. This protection is distinct for the three IPMDHs with different heat stabilities. The substrate does not cause significant increase in stability of Tt enzyme that has the highest structural rigidity and heat stability, while Ec and Vib enzymes with higher structural flexibilities can be stabilized at much higher extents. In contrast of denaturation, the rate of **renaturation process is not influenced** by the presence of substrate.

3.

The kinetic results of denaturation and renaturation are interpreted by molecular graphical analysis of the structural data. **The specific interactions among the non-conserved side-chains are suggested to be responsible for both the different denaturation rates** and the heat stabilities. These are illustrated in Fig. 1.

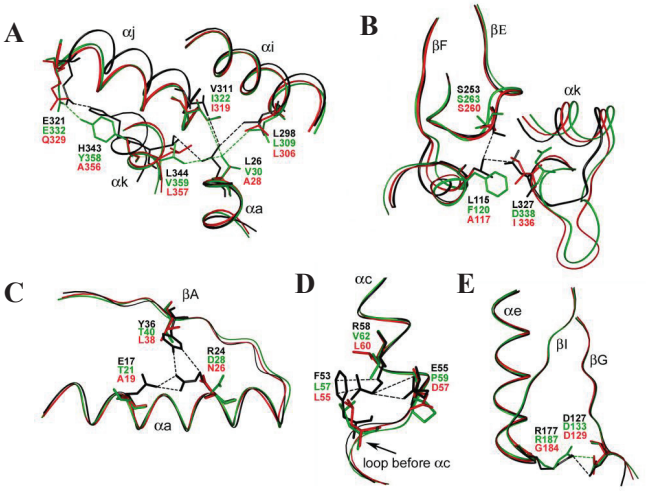

Figure 1 Structural differences between IPMDHs with different heat stabilities

The secondary structure elements are represents by black (Tt), green (Ec) and red (Vib) ribbon diagrams. The three structures were superimposed by according to the Cα atoms of all core β-strands. The non-conserved side-chains are shown as stick models in the respective colours. Dashed lines represent atomic interactions. Panels A, C and D show selected details of domain 1; panel B illustrates the interdomain region, and panel E refers to domain 2.

Of the 27 investigated non-conserved residues, as many as 16 were found where the observed differences in the atomic contacts are clearly in line with the different heat stabilities. This analysis has identified three particular regions (domain 1, interdomain region, subunit-subunit interface) of IPMDH molecule that may have the greatest influence on heat stability.

The stabilizing role of the bound **Mn*IPM** can be explained by **fixation of the relative steric positions of the secondary structural elements** that contain side-chains interacting directly with the substrate. Besides, several other conserved residues that do not contact directly with the substrate, can also participate in stabilization of the structure.

4.

In case of IPMDH no any correlation was found between the folding rate and heat stability, although such examples for other proteins are found in the literature. Thus **the specific contacts** of non-conserved residues – **responsible for the different heat stabilities of IPMDHs – are most probably formed only at some later stage(s) of folding, after the rate limiting step(s)**. In order to draw more general conclusions further renaturation experiments have to be carried out with proteins of different heat stabilities. However, decreasing the denaturation rate with increasing heat stability (as found with IPMDHs) seems to be a more general stabilizing mechanism, that was also observed in case of several hyperthermophilic proteins.

5.

Formation of an intermediate was detected during the time course of renaturation. This **intermediate** already exhibited native-like character based on Trp fluorescence measurements. The λ_{max} value of its emission spectrum is similar to that of the native enzyme, but the fluorescence emission intensity is much smaller compared to the native enzyme. Thus, partial desolvatation of the Trp residues have already occured at early stages of refolding. This is also supported by ANS fluorescence measurements, where a **molten globule-like** intermediate was detected. Additional fluorescence anisotropy experiments established that the intermediate state is presumably an associated form of the two polypeptide-chains, i.e. the intermediate represents an early **dimeric form**. The intermediate is also **able to bind the substrate**, as demonstrated by FRET measurements, however these enzyme-substrate contacts are not native-like, since the intermediate was found to be **completely inactive**.

6.

Concerning the role of subunits in formation of the native three-dimensional structure of IPMDHs, the above experimental findings clearly demonstrate the necessity of **association of the two polypeptide chains at the very early phase of refolding.** Thus, productive refolding of **the native dimer is resulted in by cooperative refolding of the two chains.**

7.

Besides the role of subunits, the role of IPMDH domains in the renaturation process was also investigated. The polypeptide chain Tt IPMDH contains 3 Trp residues, W77, W152, and W195. In the spatial structure of IPMDH W152 is positioned within the arm-like region, while W77 and W195 are located in separate domains, domain 1 and domain 2, respectively. In order to study refolding of selected areas separately within the protein molecule, I have systematically replaced the Trp residues of Tt IPMDH with much less fluorescent Phe, by site-directed mutagenesis. The Trp fluorescence and FRET measurements are unambiguously revealed, that the native structure formation of domains in the subunit takes place in an entirely symmetrical way. Thus, **two domains are folded simultaneously, in close contact with each other during the whole refolding process. Thereby, the domains facilitate formation of the native structure of each other**.

8.

The close relationship between folding of subunits and domains has been supported by analysis of atomic interactions at the subunit-subunit and domain-domain interfaces.

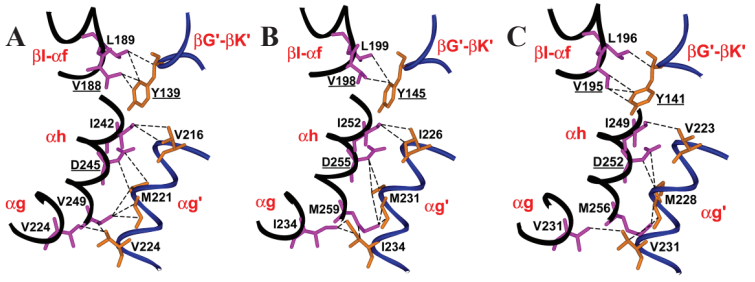

Figure 2 Comparison of the subunit contacts of IPMDHs with different heat stabilities

The subunits of Tt (A), Ec (B) and Vib (C) IPMDHs are represented by black and blue ribbon diagrams. The side-chains – participating in the subunit contacts – are shown as pink and orange stick models, respectively. Dashed lines represent atomic interactions. The labels of conservative side-chains are underlined.

Structural analysis has revealed that the **interactions of subunits and domains are mainly hydrophobic** in their nature **and are very similar in cases of the three different IPMDHs, i.e. independent of the heat stabilities** (Figure 2 and 3). These findings explain the highly cooperative mode of folding of IPMDHs with different heat stabilities. It is also notable that the residues – participating in formation of the connections between subunits or domains – are not necessarily conserved ones, their similar nature (e.g. the hydrophobic character) is sufficient.

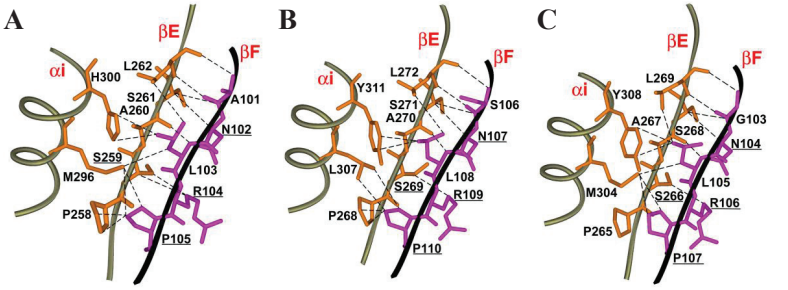

Figure 3 Comparison of contacts on the domain surfaces of IPMDHs with different heat stabilities

Parts of the domain 1 and domain 2 are represented by gray and black ribbon diagrams in the Tt (A), Ec (B) and Vib (C) IPMDHs, respectively. The side-chains – participating in the domain connection – are shown by stick models of pink and orange colours, respectively. Dashed lines represent atomic interactions. The labels of conserved side-chains are underlined.

In summary, the results of my PhD work - including various types of experiments with three different IPMDHs and molecular graphical analyses of their crystal structural data – has provided further **insight into the mechanism of formation of the native three dimensional structure of proteins possessing subunits and/or domains.** A highly cooperative folding mechanism of subunits and domains are envisaged for all investigated IPMDHs. **Furthermore, a special relationship between both unfolding and refolding processes and the heat stability has been presented.** While the folding rate and mechanism are independent of the heat stabilities of IPMDHs, having very similar folding topologies, unfolding rates are inversely proportional to their stabilities. The high stability is due to formation of specific contacts between non-conserved residues, characteristic of heat resistant proteins. Mapping of these contacts allows not only to understanding the molecular basis of structural stabilities of proteins, but also contributes to designing and production of new proteins possessing improved structural stabilities.

Publication list

1. Refereed, scientific journal publications related to the Ph. D. dissertation:

É.,Gráczer, A., Varga, B., Melnik, G., Semisotnov, P., Závodszky & M.,Vas
Symmetrical Refolding of Protein Domains and Subunits: Example of the Dimeric Two-Domain 3-Isopropylmalate Dehydrogenase
Biochemistry (2009) **48** 1123-1134

É., Gráczer, A., Varga, I., Hajdú, B., Melnik, A.,Szilágyi, G., Semisotnov, P., Závodszky & M., Vas
Rates of Unfolding, rather than Refolding, Determine Thermal Stabilities of Thermophilic, Mesophilic and Psychrotrophic 3-Isopropylmalate Dehydrogenases
Biochemistry (2007) **46** 11536-11549

2. Other refereed, scientific journal publications:

A., Varga, B., Flachner, P., Konarev, **É., Gráczer,** J., Szabó, D., Svergun, P., Závodszky, M., Vas
Substrate-induced double sided H-bond network as a means of domain closure in 3-phosphoglycerate kinase
FEBS Lett. (2006) **580** 2698-2706

A., Varga, B., Flachner, **É., Gráczer,** S., Osváth, AN., Szilágyi, M., Vas
Correlation between conformational stability of the ternary enzyme-substrate complex and domain closure of 3-phosphoglycerate kinase
FEBS Journal (2005) **272** 1867-1885

3. Conference publications, conference summaries:

(name of the lecturer of the interpretation or of the poster is the first)

É.,Gráczer, A., Varga, B., Melnik, G., Semisotnov, P., Závodszky & M., Vas
Poster title: Az alegységek és a domének kooperativitása az izopropilmalát dehidrogenáz (IPMDH) térszerkezet kialakulása során
A Magyar Biofizikai Egyesület XXIII. Kongresszusa, Pécs 2009
Konferencia kiadvány, 73. oldal

M., Vas, A., Varga, J., Szabó, **É.**, **Gráczer,** B., Flachner, P., Závodszky, P., Konarev & D., Svergun
Lecture title: Insight into the mechanism of domain movements and its role in functioning of 3-phosphoglycerate kinase
„Biocatalysis-2007. Structure, functions, application" Nemzetközi konferencia
Moszkva, Szentpétervár, Oroszország 2007
Vestnik Moscow University Bulletin, Ser. No. 2. (2008) 48 142-147

É., Gráczer, A., Varga, I., Hajdú, B., Melnik, G., Semisotnov, P., Závodszky & M., Vas
Poster title: Correlation between thermal stabilities of IPMDHs and their unfolding rates
VI. Európai Biofizikai Kongresszus, London 2007
Eur. J. Biophys. **36,** p. S159, P-413

É., Gráczer, A., Varga, I., Hajdú, B., Melnik, A., Szilágyi, G., Semisotnov, P., Závodszky & M., Vas
Lecture title: Rates of unfolding, rather than refolding determine thermal stabilities of thermophilic, mesophilic and psychrotrophic isopropylmalate dehydrogenases (IPMDH)
Straub napok, Szeged 2007

É., Gráczer, A., Varga, I., Hajdú, G., Semisotnov, P., Závodszky, & M., Vas
Poster title: Az izopropilmalát dehidrogenázok (IPMDH) hőstabilitásbeli különbségeit denaturációjuk sebessége határozza meg
A Magyar Biokémiai Egyesület Vándorgyűlése, Pécs 2006
Biokémia szeptemberi szám, 70. oldal P-29

A., Varga, **É.**, **Gráczer,** I., Hajdú, P., Závodszky, & M.,Vas
Poster title: Association of subunits is a prerequisite for formation of the native structure of the dimeric isopropylmalate-dehydrogenase (IPMDH)
V. Európai Biofizikai Kongresszus, Montpellier 2005
Eur. J. Biophys. **34**, p.796, P-839